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Functional simian immunodeficiency virus Gag-specific CD8+ intraepithelial lymphocytes in the mucosae of SIVmac251- or simian–human immunodeficiency virus KU2-infected macaques

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Abstract

The vaginal and rectal mucosae are the first line of cellular immune defense to sexually transmitted human immunodeficiency virus type 1 (HIV-1) entry. Thus, intraepithelial lymphocytes (IELs) may be important in the immune response to HIV infection. Here we investigated whether functional IELs in mucosal compartments could be visualized by direct staining with a tetrameric complex specific for the simian immunodeficiency virus (SIV) immunodominant Gag epitope in either separated IEL cells or tissues of macaques infected with SIVmac251. Of the 15 Mamu-A*01-positive macaques studied here, eight were chronically infected with either SIVmac251 or simian–human immunodeficiency virus (SHIV) KU2 and the remaining seven were exposed mucosally to SIVmac251 and sacrificed within 48 h to assess the local immune response. Gag-specific CD8+ T-cells were found in separated IELs from the rectum, colon, jejunum, and vagina of most infected animals. Direct staining of tetramers also revealed their presence in intact tissue. These Gag-specific IELs expressed the activation marker CD69 and produced IFN-γ, suggesting an active immune response in this locale.
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Keywords: CD8+ IELs; SIVmac infection; Mucosal compartments

Introduction

The assessment of immune responses to HIV at entry sites is important because mucosal sites are the targets of HIV infection. Some studies have shown that HIV-1 can be rapidly internalized by intestinal epithelial cells, transcytosed toward the basolateral pole of the cell, and released in its infectious form (Bomsel, 1997; Hocini and Bomsel, 1999). Other studies have demonstrated that HIV can cross the intact stratified epithelia of vagina, exocervix, foreskin, and anus probably by infecting processes of dendritic cells that extend into the lumen (Fantini et al., 1997; Hu et al., 2000; Spira et al., 1996).

Therefore, the presence of immune cells at these sites may help to contain viral replication early on, as a recent study has suggested (Belyakov et al., 2001). Key components of mucosal defense against infection include mucosal IgA, the anti-invasive glycocalyx of the mucosae, and the intraepithelial lymphocytes (IELs) (Lefrancois, 1999). IELs are a population of predominantly CD3+CD8+ T-cells interspersed within the epithelial layer and thus directly exposed to outside antigens (Lefrancois, 1999). Therefore, at mucosal sites, IELs represent the first available cellular line of defense against the virus. In humans, the IEL population is heterogeneous and differences among IELs are present along the length of the intestine. In the intestine of nonhuman primates, γ/δ T-cell-receptor (TCR)-positive CD3+CD8+ IELs represent only 5% of the total population and do not participate in typical cytotoxic T-lymphocyte (CTL) activity (Mattapallil et al., 1998). Other cells present are α/α TCR CD8+ T-cells that are mainly CD4+CD8+ double-positive cells that decrease during infection (Mattapallil et al., 2000)

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Table 1
Time from infection and CD4+ T-cell counts in chronically infected Mamu-A*01-positive macaques

Macaque	Time of infection (weeks)	Virus	CD3+CD4+ T-cell count	RNA copies/ml plasma at time of tissue collection
409	129	SHIV KU2	1234	<20000
450	103	SIVmac251	1165	<20000
3218	16	SIVmac251	909	Not available
3248	23	SIVmac251	345	Not available
454	156	SHIV KU2	781	<20000
455	156	SHIV KU2	956	<20000
460	127	SIVmac251	684	56290
649	124	SIVmac251	1476	401110

and α/β TCR CD8+ T-cells with antigen-specific cytolytic activity. α/β TCR CD8+ T-cells were also shown to be the main population in the vaginal epithelium of macaques (Lohman et al., 1995).

CTL activity of IELs was previously described in the vaginal epithelium and in the small intestine epithelium of simian immunodeficiency virus (SIV) chronically infected macaques (Couedel-Courteille et al., 1997; Lohman et al., 1995). SIV Gag- and Env-specific CTL activity was detected in primary cultures of IELs from macaques infected with SIV for 1, 2, and 4 weeks (Mattapallil et al., 1998), and in the same study a decrease of CD4+ IELs was also observed. A follow-up study showed that the decreased CD4+ IEL population consisted of double-positive CD8α/α+CD4+ cells. This was accompanied by an increase of CD8α/β+ IELs that are considered to be mature cells capable of mounting a CTL response (Mattapallil et al., 2000). However, all of these studies were done on cultured cells derived from IELs. Here we assessed the frequency of antigen-specific CD3+CD8α/β+ IELs in the jejunum, colon, and rectum as well as from the vaginal epithelium of SIVmac-infected macaques using the tetramer technology. Direct visualization of tetramer-positive SIV Gag-specific IELs (Altman et al., 1996) within the colon was also performed by in situ tetramer staining on tissues.

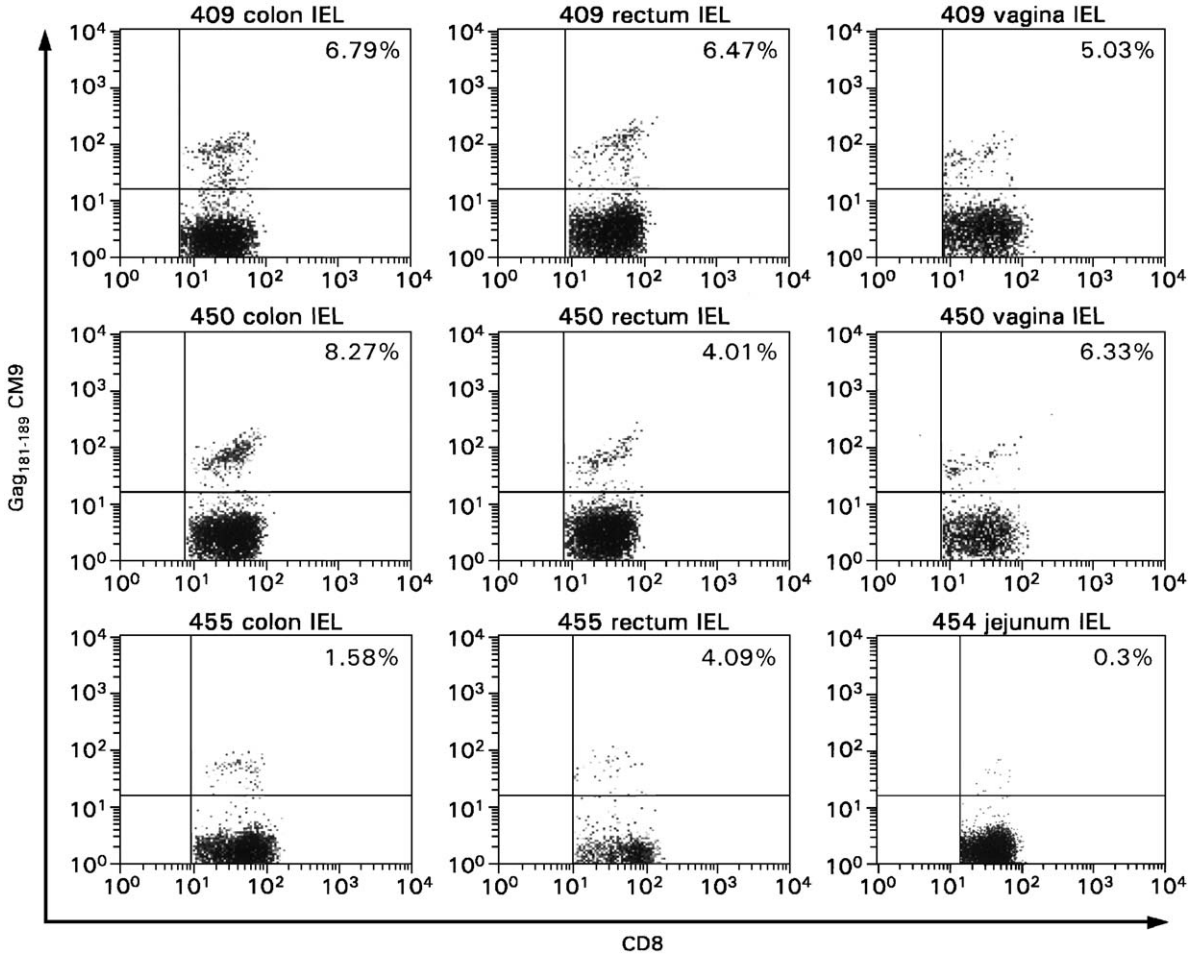

Fig. 1. Frequency of $Gag_{181-189}$ CM9 tetramer-positive cells in the IEL population of chronically SIVmac251- or SHIV KU2-infected macaques. SIV-specific IELs from the colon, rectal, and vaginal mucosae of chronically infected macaques described in Table 1. Three-color staining was done, cells were gated through the CD3+CD8+ population, and quadrant analysis was performed for expression of $Gag_{181-189}$ CM9 and CD8. CD3+CD8+ cells (1×10^4) were acquired for each analysis.

Results

High frequency of SIV Gag-specific CD8+ lymphocytes in the intraepithelial compartment of chronically SIVmac251- or SHIV KU2-infected macaques

We and others have previously shown that CD3+CD8+ T-cells recognizing the dominant $Gag_{181-189}$CM9 (p11c) peptide can be found not only in blood and lymphoid organs but also in the gut-associated lymphoid tissue (GALT) and lamina propria of Mamu-A*01-positive macaques during both primary and long-standing SIVmac infection (Hel et al., 2001; Imaoka et al., 1998; Murphey-Corb et al., 1999; Stevceva et al., 2001, 2002a, 2002b). Here, we assessed the relative frequency of CD8α/β+ lymphocytes, specific for the immunodominant SIVmac peptide Gag_{81-189}CM9, in the intraepithelial compartment of colon, jejunum, rectal, and vaginal mucosae in eight Mamu-A*01-positive macaques with longstanding SIVmac251 infection. Most of these animals had CD4+ T-cell counts within a normal range (Table 1) and were able to naturally control viral replication (i.e., plasma virus levels were less than 2000 copies/100 μl of plasma input), as previously reported (Pal et al., 2001). The percentage of CD3+CD8α/β+$Gag_{181-189}$CM9-positive tetramer-staining cells in the IELs isolated from colon, rectum, and vagina varied among animals and in some cases was as high as 8% of the total CD3+CD8+ T-cells present in these compartments, as demonstrated for three of the eight macaques in Fig. 1.

To confirm the location of Gag tetramer-positive cells within the intact epithelial layer, in situ tetramer staining was performed on ex vivo intestinal tissues from chronically infected macaque 649. As demonstrated in Fig. 2, a portion of CD3+ T-cells was also stained by the $Gag_{181-189}$CM9 tetramer, confirming their presence in the intact intestinal epithelial layer.

Functionality of Gag-specific CD8+ IELs

To assess the activation status of total and Gag-specific CD8+ T-cells in the mucosal compartments of these animals, cells were stained with an Ab that recognizes the CD69 activation marker. As demonstrated in Fig. 3A, most of the IEL cells in jejunum, colon, rectum, and vagina from macaques 460, 455, 454, 409, and 450 stained positive for CD69, demonstrating their activation status (Fig. 3A). A similar analysis of the $Gag_{181-189}$CM9-specific population in animals 450 and 409 demonstrated that most of these virus-specific CD8+ T-cells expressed the CD69 marker, indicating that the SIV-specific population of CD8α/β+IELs had an activated phenotype in colon, rectum, and vagina (Fig. 3A). These data agree with observations by others (Couedel-

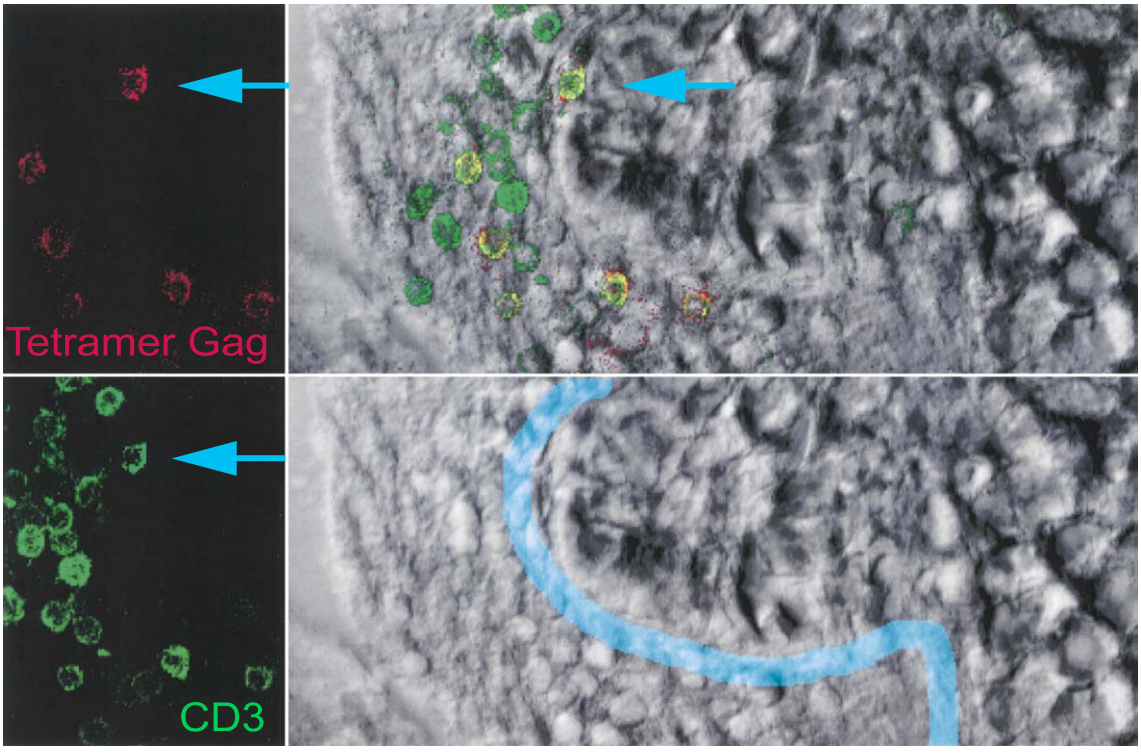

Fig. 2. In situ tetramer staining for $Gag_{181-189}$ CM9-specific IELs. Images for individual channels (CD3, green; SIV Gag tetramer, red) are shown on the left-hand side. Differential interface contrast and gray scale are shown on the bottom right panel. The basal membrane, separating the epithelial layer from the lamina propria, is labeled with a blue line. A merged image, containing all three channels, is shown on the top right panel. An example of a CD3+ IEL that is also positive for the $Gag_{181-189}$ CM9 tetramer is labeled with an arrow. FITC, fluorescein isothyocyanate, CY3, indocarbocyanine.

Courteille et al., 1997; Lohman et al., 1995; Mattapallil et al., 1998) and go along with the demonstration by others of IEL cytolytic activity. In contrast, the expression of CD25 was very low, both on the CD3+CD8+ IEL population as well as on the $Gag_{181-189}$CM9-specific IELs (data not shown).

To assess the ability of IELs to produce cytokines, in vitro stimulation with lipopolysaccharide (LPS), PMA/ionomycin, or the GagGag$_{181-189}$CM9 peptide was performed on cells from the IELs or lamina propria from various compartments. The cells were stained for intracellular

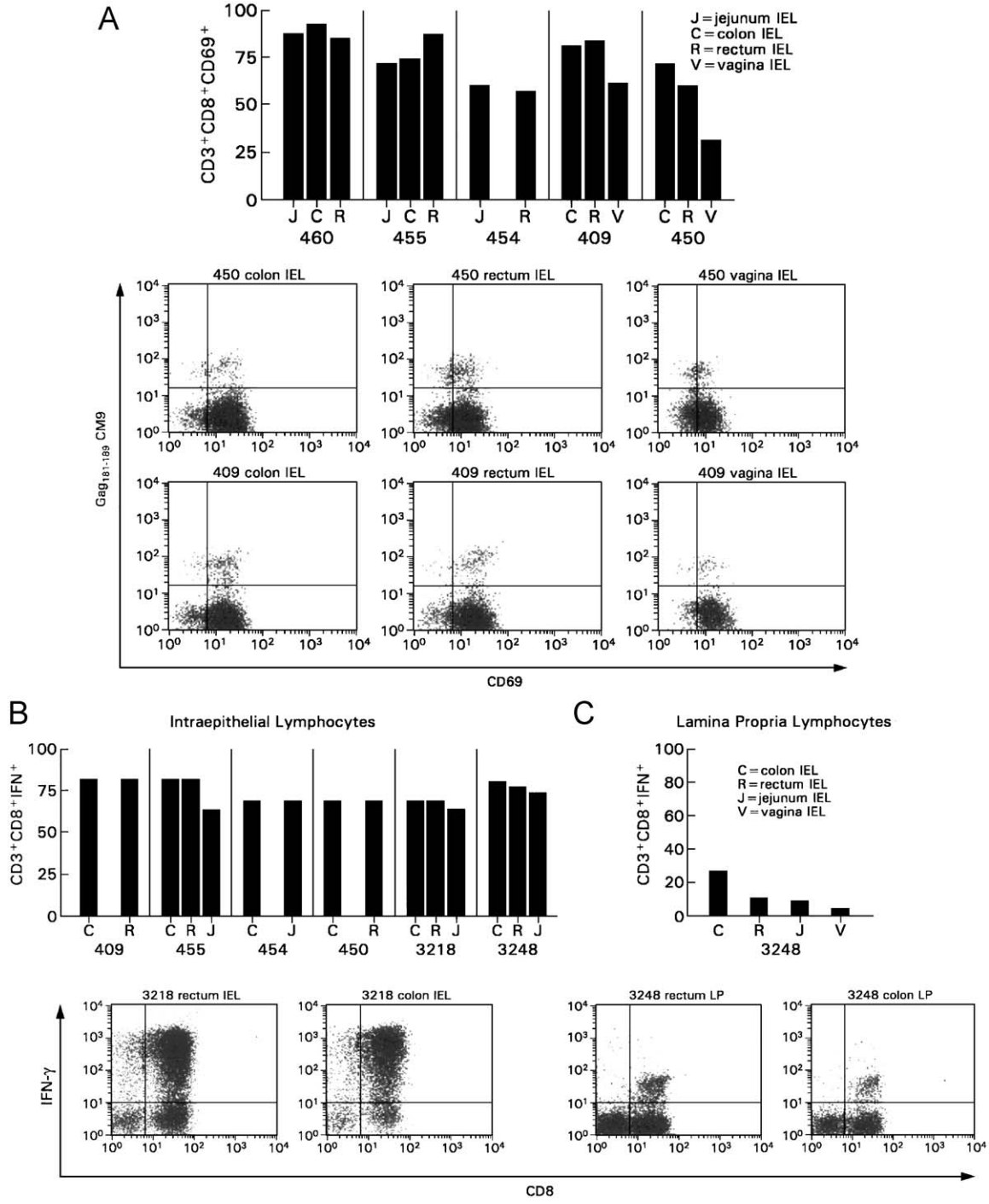

Fig. 3. Activation status and functionality of IEL and lamina propria CD8α/β+ T-cells. (A) Four-color staining was done on isolated IELs from chronically infected macaques. Cells were gated through the CD3+CD8+ population and then quadrant analysis was performed for expression of $Gag_{181-189}$ CM9 and CD69. Following unspecific stimulation with PMA and ionomycin, expression of IFN-γ was higher in the IELs (B) than in the lamina propria lymphocytes (C) from the same macaque. Raw data are demonstrated for macaques 3218 and 3248 in the bottom panels of the figure. (D) Following 24-h stimulation with LPS, intracellular cytokine staining revealed fairly low expression of the IL-10 cytokine. (E) Percentage of $Gag_{181-189}$ CM9-specific tetramer-positive cells producing IFN-γ.

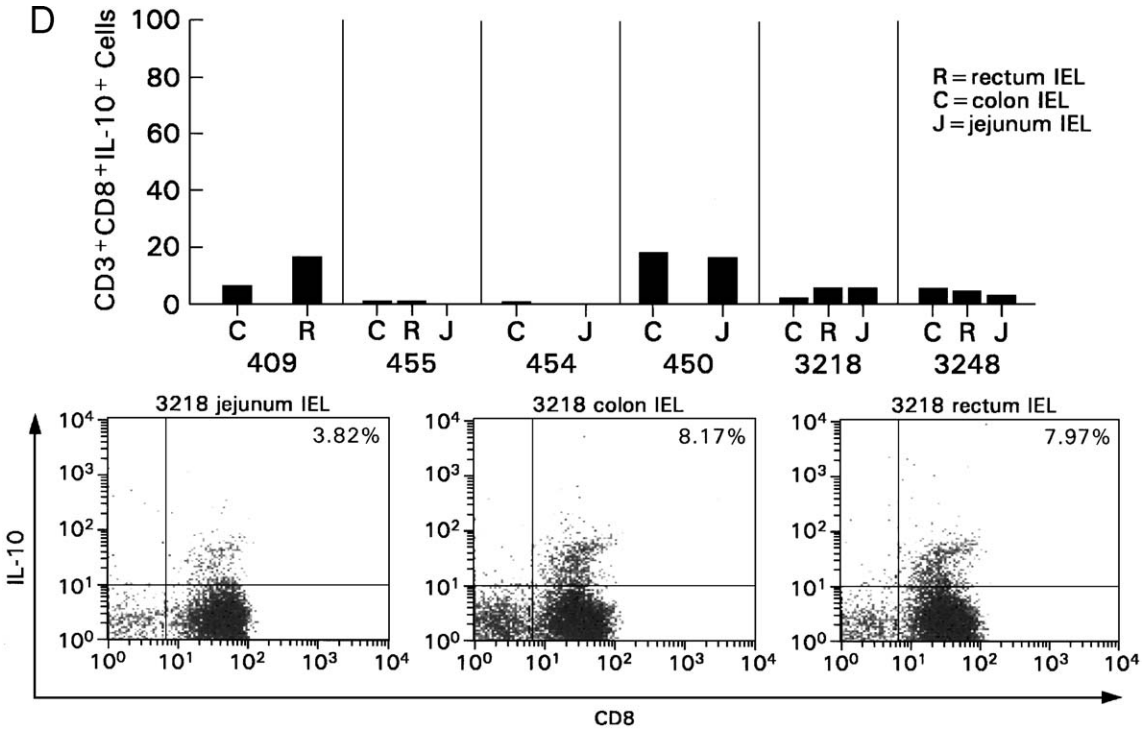

Fig. 3 (*continued*).

IFN-γ and IL-10 production. More than half of the CD8α/β+ IELs produced IFN-γ following PMA and ionomycin stimulation, demonstrating their viability (Fig. 3B). The percentage of CD8α/β+ IELs that produced IFN-γ was generally higher in the intraepithelial compartment than in the lamina propria (Fig. 3C). In contrast, following LPS stimulation, a much smaller percentage (4–19%) of IEL cells produced IL-10, a cytokine that is believed to be involved in peripheral tolerance (Chen et al., 1995; Dallman et al., 1993; Enk et al., 1993) (Fig. 3D).

Following antigen-specific stimulation, very few if any of the CD3+CD8+GagGag$_{181-189}$CM9-positive cells secreted IL-10 (range: 0.03–0.28%) (data not shown), whereas a higher proportion of these cells produced IFN-γ (Fig. 3E). The finding that only a small fraction of GagGag$_{181-189}$CM9-specific IEL cells produced IFN-γ upon antigen-specific stimulation may relate to the low percentage of IELs expressing the co-stimulatory molecules CD28 and CD49d, as reported by others (Couedel-Courteille et al., 1997; Gelfanov et al., 1995; Reimann and Rudolphi, 1995; Sillett et al., 1999).

Antigen-specific IELs in the mucosae of vaccinated macaques following SIVmac251 exposure

We have previously demonstrated that NYVAC-SIV-*gag-pol-env (gpe)* immunization of naive macaques by mucosal or systemic routes can induce virus-specific CD8+ T-cells that traffic to the lamina propria (Stevceva et al., 2002a, 2002b) and that these cells are expanded following virus encounter. Because we wished to address whether viral exposure could induce virus-specific IEL responses, the same macaques immunized with the highly attenuated recombinant NYVAC-SIV-*gpe* vaccine candidate by various routes were exposed to SIVmac251 (Stevceva et al., 2002a, 2002b) (Table 2). Animal 654 was not immunized before viral exposure and was challenged intrarectally together with animals 815, 582, 536, 818, and 814, as previously described (Stevceva et al., 2002a, 2002b). All animals were sacrificed at 48 h post-challenge and the frequency of GagGag$_{181-189}$CM9-specific tetramer-positive cells was measured in purified IELs. Tetramer-positive CD3+CD8+ IEL cells were observed in all macaques previously immunized with NYVAC-SIV-*gpe* but not in the unimmunized macaque 654 (Fig. 4). These data suggest the possibility that prior vaccination induced not only Gag-specific tetramer-positive cells in the lamina propria (Stevceva et al., 2002a, 2002b) but also IELs with the same specificity and that their frequency may be higher in immunized macaques as a result of the secondary stimulation induced by viral exposure.

Discussion

Developing a successful HIV vaccine may be greatly hindered by the difficulties in obtaining strong and durable immune responses at the sites of viral entry, including vaginal and rectal mucosae. Protection studies with vaccine candidates have so far shown only partial protection upon mucosal challenge (Belyakov et al., 2001; Benson et al.,

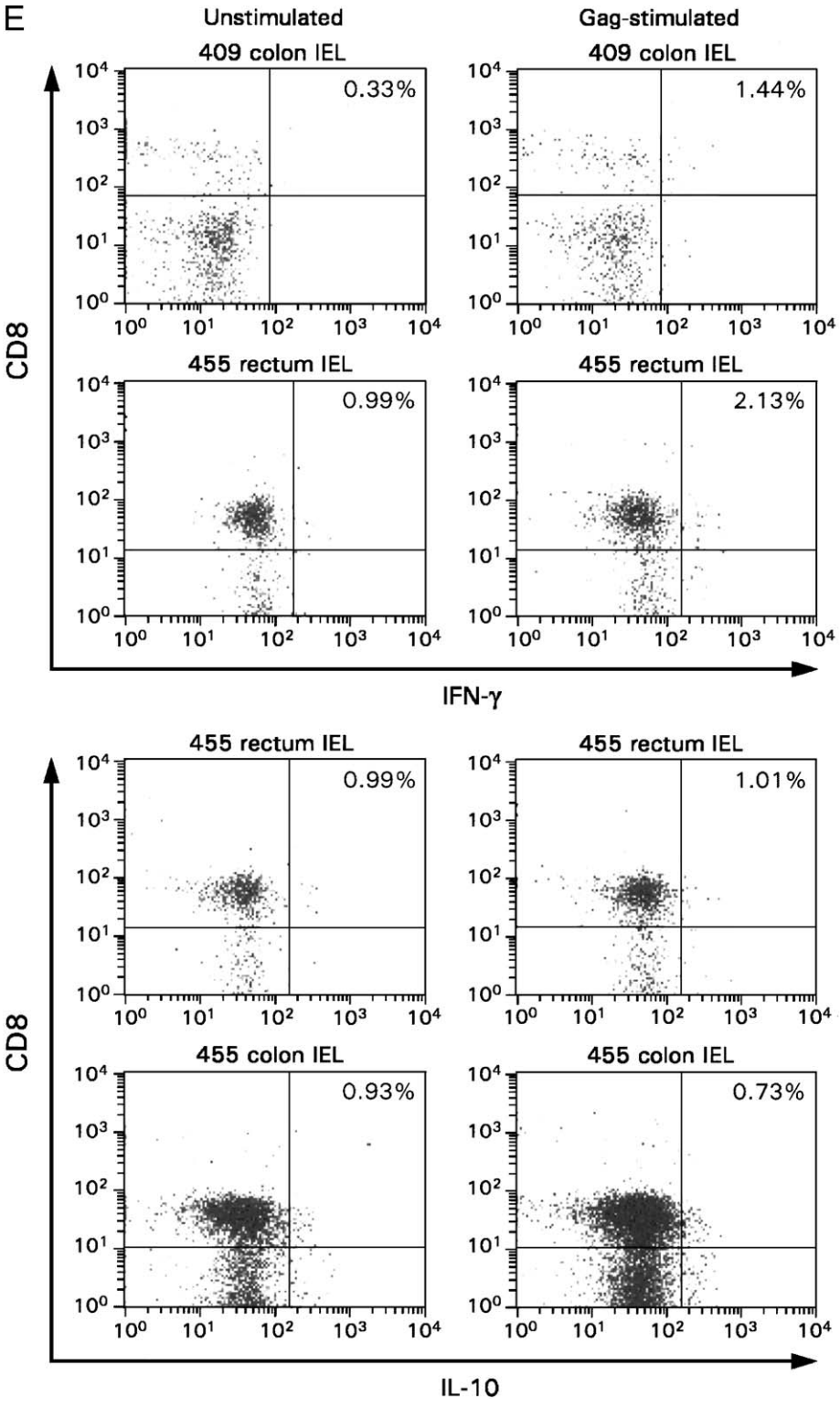

Fig. 3 (*continued*).

1998; Buge et al., 1997, 1999; Fuller et al., 2002; Hel et al., 2002; Lu et al., 1998). Efforts to obtain immune response by immunization have been focused on other arms of the immune response at mucosal surfaces, such as lamina propria lymphocytes and the readily inducible IgG and IgA responses. Very little attention has been paid to the antigen-specific IELs that are ideally located at mucosal surfaces to intercept the virus and limit its spreading. Previous studies have demonstrated cytotoxic activity in cultured lymphocytes isolated from cervicovaginal mucosae

Table 2
Immunization history of some of the Mamu-A*01-positive macaques

Macaque	Immunogen/route	CD3+CD4+ T-cell count
536	NYVAC-SIV-*gpe*/intramuscular	1668
815	NYVAC-SIV-*gpe*/intramuscular	2491
814	NYVAC-SIV-*gpe*/intrarectal	1441
818	NYVAC-SIV-*gpe*/intrarectal	1269
582	NYVAC-SIV-*gpe*/intranasal	917
654	None	1300

(Lohman et al., 1995) and intestinal epithelia (Mattapallil et al., 1998). However, direct demonstration of their presence at these sites has not been provided.

Here, by using the tetramer technology, we were able to detect the presence of SIV Gag-specific lymphocytes in the intraepithelial compartment of intestinal and vaginal mucosae on isolated IELs and in tissues of SIVmac251- or simian–human immunodeficiency virus (SHIV) KU2-infected macaques. Furthermore, we demonstrated that these cells are activated and able to secrete IFN-γ.

The generation of Ag-specific IELs that exert cytotoxic activity upon ex vivo isolation has been reported in studies by others in SIV-infected macaques as well as in other infections such as rotavirus (Dharakul et al., 1990; Offit and Dudzik, 1989) and lymphocytic choriomeningitis virus (LCMV) (Muller et al., 2000; Sydora et al., 1996). Studies in mice have demonstrated that Ag-specific IELs are capable of inducing protective immunity. Passive transfer of isolated primed IELs from mice infected with *Toxoplasma gondii* cysts containing bradyzoites provides long-term protection by reducing the cyst numbers and results in survival of all mice if transferred 6–9 days postinfection (Lepage et al., 1998). The protective cells were shown to be CD8α/β+ (Buzoni-Gatel et al., 1997). Furthermore, in this model, it has been demonstrated that it is possible to induce these protective IELs by intranasal immunization with the SAG1 protein of *T. gondii* (Velge-Roussel et al., 2000).

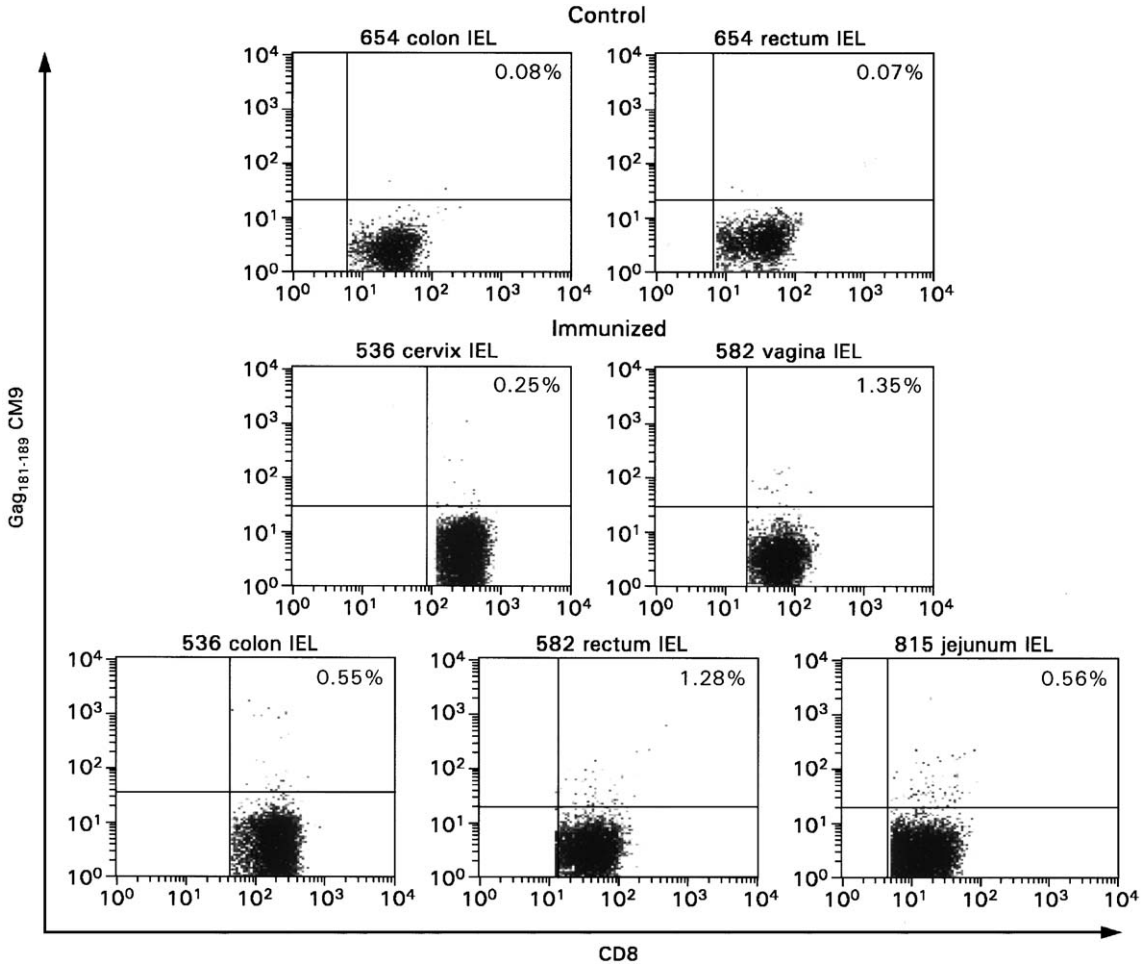

Fig. 4. Detection of Gag-specific CD8α/β+ T-cells early in post-viral exposure. In the naive macaque 654, the IEL level detected with the $Gag_{181-189}$ CM9 tetramer was below 1%. CD3+CD8+ $Gag_{181-189}$ CM9-positive IELs were found in the cervicovaginal and intestinal tissues of macaques immunized before SIVmac251 challenge (536, 582, and 815). Isolated IELs were initially gated through the CD3+CD8+ population. Percentage of CD3+CD8+ $Gag_{181-189}$ CM9-positive cells is shown in the upper right quadrant. CD3+CD8+ cells (1×10^4) were acquired for each analysis.

Similarly, gut CD4+ IELs were shown to be able to induce protection against *Cryptosporidium muris* when adoptively transferred to immunocompromised SCID mice (McDonald et al., 1996). In both of these cases, protection was shown to be mediated by IFN-γ (Buzoni-Gatel et al., 1997; Culshaw et al., 1997).

The results of the present study demonstrate the presence of SIV Gag-specific IELs in ex vivo samples of nonhuman primates infected with a lentivirus related to HIV-1. Furthermore, we have shown that SIV Gag-specific IELs are activated and able to secrete high levels of IFN-γ, a cytokine that has been related to protection (Heeney et al., 1998; Kuhn et al., 2001). Others have shown that in SIVmac infection virus-specific IELs are able to lyse cells expressing the SIVmac Gag and Env antigens (Couedel-Courteille et al., 1997; Lohman et al., 1995; Mattapallil et al., 1998). Even though it has been shown that the tetramer-binding assay detects more HIV-specific CD8+ T-cells than other methods (Sun et al., 2003) and that not all tetramer-binding cells seem to produce IFN-γ (Appay et al., 2000; Goepfert et al., 2000; Hel et al., 2001; Shankar et al., 2000), it does not require expansion of cells in culture and can be performed on a few cells. Thus, in light of these findings, quantitation of Ag-specific IELs at mucosal sites may become a feasible assay in vaccination studies and contribute to the understanding of correlates of protection or viremia containment.

Materials and methods

Animals and procedure

A total of 14 Mamu-A*01-positive rhesus macaques (*Macaca mulatta*) were studied. Five animals (450, 3218, 3248, 460, and 649) were chronically infected with SIVmac251 (Pal et al., 2001) and three animals (409, 454, and 455) with SHIV KU2, a chimeric virus that expresses the SIVmac Gag protein (Table 1). Of the remaining six macaques, five (815, 536, 582, 814, and 818) were first immunized with NYVAC-SIV-*gpe*, exposed to SIVmac251 (561), and sacrificed 48 h later (Stevceva et al., 2002a, 2002b) (Table 2), and one, 654, was unimmunized and exposed to SIVmac251 at the same time. Among the latter group of animals, macaques 536 and 815 were first immunized with three inoculations of 1×10^8 pfu of NYVAC-SIV-*gpe* by the intramuscular route. Animal 582 was immunized intranasally and animals 814 and 818 were immunized intrarectally with three inoculations of 5×10^8 pfu of NYVAC-SIV-*gpe*. All six immunized macaques and a control naive macaque (654) were exposed to undiluted SIVmac251 (561) (Pal et al., 2001) by the intrarectal route and sacrificed 48 h after infection (Stevceva et al., 2002a, 2002b). Tissues were collected postmortem in RPMI/10% FBS and IELs were isolated as described below.

Isolation of tissue lymphocytes

Mononuclear cells were isolated from intestines, vagina, and cervix. Tissues from jejunum, colon, rectum, vagina, and cervix were treated with 1 mM DTT (ICN Biomedicals Inc., Aurora, OH) for 30 min followed by incubation in calcium/magnesium-free HBSS (Life Technologies, Baltimore, MD) three (intestines) to four (vagina and cervix) times for 1 h with stirring at room temperature to remove the epithelial layer. At this stage, pieces of tissue were fixed in 10% neutral formalin, embedded in paraffin, and sections cut and stained with H and E. We performed microscopic examination of the samples to ensure that most of the epithelium was removed and the lamina propria was intact. IELs were further purified by Percoll density gradient centrifugation.

Lamina propria lymphocytes were isolated following cutting of tissues into smaller pieces and incubated at 37 °C in Isocove's medium supplemented with 10% FCS and penicillin/streptomycin containing 400 U/ml Collagenase D (Boehringer Mannheim GmbH, Mannheim, Germany) and 25 U/ml DNAse (Worthington Biochemical Corporation, Lakewood, NJ) for 2–3 h. Vaginal and cervical tissues were digested by using Collagenase type IV (Sigma, St. Louis, MO). The mononuclear cells were isolated from the supernatant containing dissociated cells by Percoll gradient centrifugation.

Phenotypic characterization and flow cytometry

Phenotypic characterization was done by direct three- and four-color staining of freshly isolated IELs using a panel of Abs in conjunction with the tetrameric complexes (see Table 2). Staining with unrelated tetramer and of cells isolated from Mamu-A*01-negative or naive animals was used as a negative control. Staining in conjunction with CD4PE (Becton Dickinson, San Jose, CA) was used as a positive control.

Briefly, 5×10^5 lymphocytes isolated by Ficoll diatrizoate or Percoll gradient centrifugation were incubated with 2 μg of tetrameric complexes or selected Abs for 30 min at room temperature. After washing the cells twice in Dulbecco's phosphate-buffered saline supplemented with 2% FCS and fixation in 1% paraformaldehyde (Ph = 7.4), samples were analyzed by flow cytometry using the CellQuest software (CellQuest, Clearwater, FL) and the FACScalibur (Becton Dickinson) instrument.

Antigen-specific stimulation of IFN-γ and IL-10 in IEL cells

Isolated IELs in 10% FBS, penicillin/streptomycin RPMI were incubated for 4 h at 37 °C in 48-well plates at a concentration of 2×10^6 cells/well and were activated by adding either the Gag-specific peptide GagGag$_{181-189}$CM9 as described (Stevceva et al., 2002a, 2002b) in the presence of 1 μl/ml Golgiplug (Pharmingen, San Diego, CA) or 25 ng/ml PMA and 1 μg/ml ionomycin. To stimulate IL-10

production, IELs in 10% FBS, penicillin/streptomycin RPMI were incubated for 12 h at 37 °C in 48-well plates at a concentration of 2×10^6 cells/well in the presence of 1 ng/ml of LPS and 1 μl/ml Golgiplug. Cells were than stained first with surface Abs and then for intracellular IL-10 (BD Pharmingen) or IFN-γ (BD Pharmingen) by using Cytofix/Cytoperm Plus kit (Pharmingen) according to the manufacturer's instructions. HICK-1 cells (Pharmingen) were used as a positive control for IFN-γ and HICK-2 (Pharmingen) for IL-10. Unstimulated cells and cells stained only with surface markers or cells stained with isotype control were used as negative controls. Reading and flow-cytometric analysis were done using the CellQuest software and the FACScalibur (Becton Dickinson) instrument.

Tetramer staining in situ

In situ tetramer staining was performed on fresh tissues as previously described with some modifications (Haanen et al., 2000; Schmitz et al., 2001; Skinner et al., 2000; Stevceva et al., 2002a, 2002b). Briefly, tissues were collected by pinch biopsy, washed in cold PBS, and cut into small strips. The resulting sections ($n = 4–6$) were then incubated with 10 μl/section of antigen-specific tetramer labeled with Cy3 (Amersham, Piscataway, NJ) and gently agitated at 37 °C for 15 min. The tissue was then rinsed repeatedly at 37 °C with PBS and then twice with ice-cold PBS before fixation with cold 2% paraformaldehyde for 20 min. After additional washes, Abs to CD3 (Dako, Carpinteria, CA, rabbit polyclonal) and CD8 (directly conjugated to FITC, Becton Dickinson) were applied singly or together for 1 h. The tissues were then washed and incubated with anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR) when using only CD3 or anti-rabbit Alexa 568 when using both CD3 and CD8. Tissues were then washed, mounted on a glass slide with anti-fading medium (Vectashield, Vector Laboratories, Burlingame, CA), and examined by confocal microscopy.

Confocal microscopy was performed using a Leica TCS SP laser scanning microscope equipped with three lasers (Leica Microsystems, Exton, PA). Individual optical slices represent 0.2 μm and 20–60 optical slices were collected at 512 × 512 pixel resolution. The fluorescence of individual fluorochromes was captured separately in sequential mode, after optimization to reduce bleed-through between channels (photomultiplier tubes) using Leica software. NIH-image v1.62 and Adobe Photoshop v6 (Seattle, WA) software were used to assign colors to each fluorochrome and the differential interference contrast image (gray scale). Co-localization of antigens is indicated by the addition of colors as indicated in figure legends.

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References

Altman, J.D., Moss, P.A.H., Goulder, P.J.R., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., Davis, M.M., 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science 274, 94–96.

Appay, V., Nixon, D.F., Donahoe, S.M., Gillespie, G.M.A., Dong, T., King, A., Ogg, G.S., Spiegel, H.M.L., Conlon, C., Spina, C.A., Havlir, D.V., Richman, D.D., Waters, A., Easterbrook, P., McMichael, A.J., Rowland-Jones, S.L., 2000. HIV-specific CD8+T cells produce antiviral cytokines but are impaired in cytolytic function. J. Exp. Med. 192, 63–75.

Belyakov, I.M., Hel, Z., Kelsall, B., Kuznetsov, V.A., Ahlers, J.D., Nacsa, J., Watkins, D.I., Allen, T.M., Sette, A., Altman, J., Woodward, R., Markham, P.D., Clements, J.D., Franchini, G., Strober, W., Berzofsky, J.A., 2001. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. Nat. Med. 7, 1320–1326.

Benson, J., Chougnet, C., Robert-Guroff, M., Montefiori, D., Markham, P.D., Shearer, G.M., Gallo, R.C., Cranage, M.P., Paoletti, E., Limbach, K., Venzon, D., Tartaglia, J., Franchini, G., 1998. Recombinant vaccine-induced protection against the highly pathogenic SIV_{mac251}: dependence on route of challenge exposure. J. Virol. 72, 4170–4182.

Bomsel, M., 1997. Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. Nat. Med. 3, 42–47.

Buge, S.L., Richardson, E., Alipanah, S., Markham, P.D., Cheng, S., Kalyan, N., Miller, C.J., Lubeck, M., Udem, S., Eldridge, J., Robert-Guroff, M., 1997. An adenovirus-simian immunodeficiency virus *env* vaccine elicits humoral, cellular, and mucosal immune responses in rhesus macaques and decreases viral burden following vaginal challenge. J. Virol. 71, 8531–8541.

Buge, S.L., Murty, L., Arora, K., Kalyanaraman, V.S., Markham, P.D., Richardson, E.S., Aldrich, K., Patterson, L.J., Miller, C.J., Cheng, S.M., Robert-Guroff, M., 1999. Factors associated with slow disease progression in macaques immunized with an adenovirus-simian immunodeficiency virus (SIV) envelope priming—gp120 boosting regimen and challenged vaginally with SIVmac251. J. Virol. 73, 7430–7440.

Buzoni-Gatel, D., Lepage, A.C., Dimier-Poisson, I.H., Bout, D.T., Kasper, L.H., 1997. Adoptive transfer of gut intraepithelial lymphocytes protects against murine infection with *Toxoplasma gondii*. J. Immunol. 158, 5883–5889.

Chen, Y., Inobe, J., Marks, R., Gonnella, P., Kuchroo, V.K., Weiner, H.L., 1995. Peripheral deletion of antigen-reactive T cells in oral tolerance. Nature 376, 177–180.

Couedel-Courteille, A., Le Grand, R., Tulliez, M., Guillet, J.G., Venet, A., 1997. Direct ex vivo simian immunodeficiency virus (SIV)-specific cytotoxic activity detected from small intestine intraepithelial lymphocytes of SIV-infected macaques at an advanced stage of infection. J. Virol. 71, 1052–1057.

Culshaw, R.J., Bancroft, G.J., McDonald, V., 1997. Gut intraepithelial lymphocytes induce immunity against Cryptosporidium infection through a mechanism involving gamma interferon production. Infect. Immun. 65, 3074–3079.

Dallman, M.J., Wood, K.J., Hamano, K., Bushell, A.R., Morris, P.J., Wood, M.J., Charlton, H.M., 1993. Cytokines and peripheral tolerance to alloantigen. Immunol. Rev. 133, 5–18.

Dharakul, T., Rott, L., Greenberg, H.B., 1990. Recovery from chronic rotavirus infection in mice with severe combined immunodeficiency: virus clearance mediated by adoptive transfer of immune CD8+T lymphocytes. J. Virol. 64, 4375–4382.

Enk, A.H., Angeloni, V.L., Udey, M.C., Katz, S.I., 1993. Inhibition of

Langerhans cell antigen-presenting function by IL-10. A role for IL-10 in induction of tolerance. J. Immunol. 151, 2390–2398.
Fantini, J., Yahi, N., Tourres, C., Delezay, O., Tamalet, C., 1997. HIV-1 transmission across the vaginal epithelium. AIDS 11, 1663–1664.
Fuller, D.H., Rajakumar, P.A., Wilson, L.A., Trichel, A.M., Fuller, J.T., Shipley, T., Wu, M.S., Weis, K., Rinaldo, C.R., Haynes, J.R., Murphey-Corb, M., 2002. Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. J. Virol. 76, 3309–3317.
Gelfanov, V., Lai, Y.G., Gelfanova, V., Dong, J.Y., Su, J.P., Liao, N.S., 1995. Differential requirement of CD28 costimulation for activation of murine CD8+ intestinal intraepithelial lymphocyte subsets and lymph node cells. J. Immunol. 155, 76–82.
Goepfert, P.A., Bansal, A., Edwards, B.H., Ritter, G.D., Tellez, I., McPherson, S.A., Sabbaj, S., Mulligan, M.J., 2000. A significant number of human immunodeficiency virus epitope-specific cytotoxic T lymphocytes detected by tetramer binding do not produce gamma interferon. J. Virol. 74, 10249–10255.
Haanen, J.B., van Oijen, M.G., Tirion, F., Oomen, L.C., Kruisbeek, A.M., Vyth-Dreese, F.A., Schumacher, T.N., 2000. In situ detection of virus- and tumor-specific T-cell immunity. Nat. Med. 6, 1056–1060.
Heeney, J.L., van Gils, M.E., van der, M.P., de Giuli, M.C., Ghioni, C., Gimelli, M., Raddelli, A., Davis, D., Akerblom, L., Morein, B., 1998. The role of type-1 and type-2 T-helper immune responses in HIV-1 vaccine protection. J. Med. Primatol. 27, 50–58.
Hel, Z., Nacsa, J., Kelsall, B., Tsai, W.P., Letvin, N., Parks, R.W., Tryniszewska, E., Picker, L., Lewis, M.G., Edghill-Smith, Y., Moniuszko, M., Pal, R., Stevceva, L., Altman, J.D., Allen, T.M., Watkins, D., Torres, J.V., Berzofsky, J.A., Belyakov, I.M., Strober, W., Franchini, G., 2001. Impairment of Gag-specific CD8(+) T-cell function in mucosal and systemic compartments of simian immunodeficiency virus mac251- and simian–human immunodeficiency virus KU2- infected macaques. J. Virol. 75, 11483–11495.
Hel, Z., Nacsa, J., Tryniszewska, E., Tsai, W.P., Parks, R.W., Montefiori, D.C., Felber, B.K., Tartaglia, J., Pavlakis, G.N., Franchini, G., 2002. Containment of simian immunodeficiency virus infection in vaccinated macaques: correlation with the magnitude of virus-specific pre- and postchallenge CD4(+) and CD8(+) T cell responses. J. Immunol. 169, 4778–4787.
Hocini, H., Bomsel, M., 1999. Infectious human immunodeficiency virus can rapidly penetrate a tight human epithelial barrier by transcytosis in a process impaired by mucosal immunoglobulins. J. Infect. Dis. 179, S448–S453.
Hu, J., Gardner, M.B., Miller, C.J., 2000. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. J. Virol. 74, 6087–6095.
Imaoka, K., Miller, C.J., Kubota, M., McChesney, M.B., Lohman, B., Yamamoto, M., Fujihashi, K., Someya, K., Honda, M., McGhee, J.R., Kiyono, H., 1998. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. J. Immunol. 161, 5952–5958.
Kuhn, L., Coutsoudis, A., Moodley, D., Mngqundaniso, N., Trabattoni, D., Shearer, G.M., Clerici, M., Coovadia, H.M., 2001. Interferon-gamma and interleukin-10 production among HIV-1-infected and uninfected infants of HIV-1-infected mothers. Pediatr. Res. 50, 412–416.
Lefrancois, L., 1999. Basic aspects of intraepithelial lymphocyte immunobiology. In: Ogra, P.L., Mestecky, J., Lamm, M.E., Strober, W., Bienenstock, J., McGhee, J.R. (Eds.), Mucosal immunology. Academic Press, San Diego, pp. 413–428.
Lepage, A.C., Buzoni-Gatel, D., Bout, D.T., Kasper, L.H., 1998. Gut-derived intraepithelial lymphocytes induce long term immunity against *Toxoplasma gondii*. J. Immunol. 161, 4902–4908.
Lohman, B.L., Miller, C.J., McChesney, M.B., 1995. Antiviral cytotoxic T lymphocytes in vaginal mucosa of simian immunodeficiency virus-infected rhesus macaques. J. Immunol. 155, 5855–5860.
Lu, X., Kiyono, H., Lu, D., Kawabata, S., Torten, J., Srinivasan, S., Dailey, P.J., McGhee, J.R., Lehner, T., Miller, C.J., 1998. Targeted lymph-node immunization with whole inactivated simian immunodeficiency virus (SIV) or envelope and core subunit antigen vaccines does not reliably protect rhesus macaques from vaginal challenge with SIVmac251. AIDS 12, 1–10.
Mattapallil, J.J., Smit-McBride, Z., McChesney, M., Dandekar, S., 1998. Intestinal intraepithelial lymphocytes are primed for gamma interferon and MIP-1beta expression and display antiviral cytotoxic activity despite severe CD4(+) T-cell depletion in primary simian immunodeficiency virus infection. J. Virol. 72, 6421–6429.
Mattapallil, J.J., Reay, E., Dandekar, S., 2000. An early expansion of CD8alphabeta T cells, but depletion of resident CD8alphaalpha T cells, occurs in the intestinal epithelium during primary simian immunodeficiency virus infection. AIDS 14, 637–646.
McDonald, V., Robinson, H.A., Kelly, J.P., Bancroft, G.J., 1996. Immunity to *Cryptosporidium muris* infection in mice is expressed through gut CD4+intraepithelial lymphocytes. Infect. Immun. 64, 2556–2562.
Muller, S., Buhler-Jungo, M., Mueller, C., 2000. Intestinal intraepithelial lymphocytes exert potent protective cytotoxic activity during an acute virus infection. J. Immunol. 164, 1986–1994.
Murphey-Corb, M., Wilson, L.A., Trichel, A.M., Roberts, D.E., Xu, K., Okhawa, S., Woodson, B., Bohm, R., Blanchard, J., 1999. Selective induction of protective MHC class I-restricted CTL in the intestinal lamina propria of rhesus monkeys by transient SIV infection of the colonic mucosa. J. Immunol. 162, 540–549.
Offit, P.A., Dudzik, K.I., 1989. Rotavirus-specific cytotoxic T lymphocytes appear at the intestinal mucosal surface after rotavirus infection. J. Virol. 63, 3507–3512.
Pal, R., Venzon, D., Letvin, N.L., Santra, S., Montefiori, D.C., Miller, N.R., Tryniszewska, E., Lewis, M.G., Vancott, T.C., Hirsch, V., Woodward, R., Gibson, A., Grace, M., Dobratz, E., Markham, P.D., Hel, Z., Nacsa, J., Klein, M., Tartaglia, J., Franchini, G., 2001. ALVAC-SIV-gag-pol-env-based vaccination and macaque major histocompatibility complex class I (A*01) delay simian immunodeficiency virus SIV(mac)-induced Immunodeficiency. J. Virol. 76, 292–302.
Reimann, J., Rudolphi, A., 1995. Co-expression of CD8 alpha in CD4+T cell receptor alpha beta+ T cells migrating into the murine small intestine epithelial layer. Eur. J. Immunol. 25, 1580–1588.
Schmitz, J.E., Veazey, R.S., Kuroda, M.J., Levy, D.B., Seth, A., Mansfield, K.G., Nickerson, C.E., Lifton, M.A., Alvarez, X., Lackner, A.A., Letvin, N.L., 2001. Simian immunodeficiency virus (SIV)-specific cytotoxic T lymphocytes in gastrointestinal tissues of chronically SIV-infected rhesus monkeys. Blood 98, 3757–3761.
Shankar, P., Russo, M., Harnisch, B., Patterson, M., Skolnik, P., Lieberman, J., 2000. Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection. Blood 96, 3094–3101.
Sillett, H.K., Southgate, J., Howdle, P.D., Trejdosiewicz, L.K., 1999. Expression of activation and costimulatory elements by human intestinal intraepithelial lymphocytes. Scand. J. Immunol. 50, 52–60.
Skinner, P.J., Daniels, M.A., Schmidt, C.S., Jameson, S.C., Haase, A.T., 2000. Cutting edge: in situ tetramer staining of antigen-specific T cells in tissues. J. Immunol. 165, 613–617.
Spira, A.I., Marx, P.A., Patterson, B.K., Mahoney, J., Koup, R.A., Wolinsky, S.M., Ho, D.D., 1996. Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. J. Exp. Med. 183, 215–225.
Stevceva, L., Tryniszewska, E., Hel, Z., Nacsa, J., Kelsall, B., Washington, P.R. and Franchini, G. (2001). Differences in time of virus appearance in the blood and virus-specific immune responses in intravenous and intrarectal primary SIVmac251 infection of rhesus macaques; a pilot study. BMC Infect Dis EDAT-2001/08/16 10:00 MHDA-2001/08/16 10:00 PHST-2001/May/10 [received] PHST-2001/Jul/27 [in press] PHST-2001/Jul/27 [aheadofprint] PST- ppublish 1, 9.
Stevceva, L., Alvarez, X., Lackner, A.A., Tryniszewska, E., Kelsall, B., Nacsa, J., Tartaglia, J., Strober, W., Franchini, G., 2002a. Both mucosal and systemic routes of immunization with the live, attenuated NY-

VAC/simian immunodeficiency virus SIV(gpe) recombinant vaccine result in gag-specific CD8(+) T-cell responses in mucosal tissues of macaques. J. Virol. 76, 11659–11676.

Stevceva, L., Kelsall, B., Nacsa, J., Moniuszko, M., Hel, Z., Tryniszewska, E., Franchini, G., 2002b. Cervicovaginal lamina propria lymphocytes: phenotypic characterization and their importance in cytotoxic T-lymphocyte responses to simian immunodeficiency virus SIV(mac251). J. Virol. 76, 9–18.

Sun, Y., Iglesias, E., Samri, A., Kamkamidze, G., Decoville, T., Carcelain, G., Autran, B., 2003. A systematic comparison of methods to measure HIV-1 specific CD8 T cells. J. Immunol. Methods 272, 23–34.

Sydora, B.C., Jamieson, B.D., Ahmed, R., Kronenberg, M., 1996. Intestinal intraepithelial lymphocytes respond to systemic lymphocytic choriomeningitis virus infection. Cell. Immunol. 167, 161–169.

Velge-Roussel, F., Marcelo, P., Lepage, A.C., Buzoni-Gatel, D., Bout, D.T., 2000. Intranasal immunization with *Toxoplasma gondii* SAG1 induces protective cells into both NALT and GALT compartments. Infect. Immun. 68, 969–972.